Degradability of crosslinked albumin as an arterial polyester prosthesis coating in *in vitro* and *in vivo* rat studies

Saïdi Benslimane, Robert Guidoin*, Paul-Emile Roy, Juan Friede, Jacques Hébert
Departments of Surgery and Pathology at Laval University, Biomaterials and Nuclear Medicine at St-François d'Assise Hospital and Immunology at CHUL Québec Canada

Dominique Domurado and Marie-Françoise Sigot-Luizard
Laboratoires de Technologie Enzymatique et de Biologie Cellulaire, Université de Compiègne, France
(Received 18 March 1985; accepted 15 September 1985)

In order to avoid the preclotting procedure in knitted polyester arterial prostheses and in woven models, compound polyester grafts have been proposed, containing preadsorbed collagen or albumin. Since we are currently investigating grafts impregnated with crosslinked albumin, it was decided to establish the degradation rate of this coating after stabilization with either glutaraldehyde (GA) or carbodiimide (CDI). Tests were performed *in vitro* by incubation in either PBS, plasma or pancreatin and *in vivo* by implantation in the abdominal cavity of rats. In PBS or plasma *in vitro*, the coatings were very stable (2% degradation after 144 h incubation), however, in pancreation the CDI crosslinked albumin degraded much faster than the GA crosslinked albumin (more than 50% degradation in 12 h compared to less than 30% in 48 h). *In vivo* the degradation rates of the two types of crosslinked albumin were similar (almost all of the albumin having been lost after 4 weeks) but the cellular response was very different: a mild tissue reaction was observed with the CDI crosslinked coating whereas many foreign body giant cells were present on the GA crosslinked material.

Keywords: Prostheses, crosslinked albumin, arterial grafts, glutaraldehyde, carbodiimide, polyester, preclotting

The preclotting of arterial polyester prostheses is preferred by most surgeons for knitted structures[1] and it is recommended for woven structures[2], prior to implantation. The wall of the blood conduit must be made impervious by impregnation of its structure with a well-penetrating and anchored thrombotic matrix to prevent blood oozing. This operation is time-consuming. The quality of the resulting flow surface depends upon the graft structure and the blood properties of the patient. This manipulation prolongs the duration of the anaesthesia and increases the risks of bacteremic colonization[3]. The resulting flow surface can lead to structures likely to embolize[4]. These drawbacks can be limited in the compound prosthesis. Bascom[5], Humphries *et al.*[6] and Jordan *et al.*[7] developed collagen-coated grafts in the early sixties. Such a concept was reintroduced by Chvapil and Krajicek[8] and led to a commercial product (Hemashield, Meadox Medicals, Oakland, NJ, USA). Based on the antithrombogenecity and cytocompatibility of albumin coatings[9], polyester prostheses were impregnated with crosslinked albumin according to a protocol proposed by Gyurko *et al.*[10] and Domurado *et al.*[11,12]. Such a graft does not require any preclotting.

*Correspondence to: Dr Robert Guidoin, Laboratory of Experimental Surgery, Faculty of Medicine, Laval University, Québec, G1K 7P4, Canada.

Histologic observations made on glutaraldehyd (GA) crosslinked albuminated grafts implanted in the thoracic aorta of dogs for 1 month do not show any albumin remaining[13]. The focus of the present investigation was t quantify *in vitro* (in saline, plasma and pancreatin) and *in vivo* (in rats) the degradation rate of albumin crosslinked by means of either glutaraldehyde or carbodiimide.

MATERIALS AND METHODS

In vitro tests

Graft selection. We selected a woven graft (Wov n d Bakey; Bard Implants Division, Billerica, MA, USA) and a texturized warp knitted graft (Vasculour II; Bard Implants). The grafts were cut into 1 cm lengths and coated according to the procedures which follow.

Albumin coating. We used two different crosslinking ag nts: glutaraldehyde (GA) (Merck, Darmstadt, FGR) and carbodiimide (CDI) [1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride]; (Sigma, St Louis, MO, USA). The grafts were soaked in the following solution: 1.1 ml of bovine serum albumin (Sigma), containing 5 μCi of ^{125}I-albumin (Frost, Kirkland, PQ), 1 ml f phosphat buff red

saline (0.5 M, pH 7.5) and 0.15 ml (0.5 M, pH 7.5) of 25% GA. The grafts were removed from the solution prior to complete gelation and the crosslinking reaction was allowed to continue overnight at room temperature. After thorough rinsing with distilled water, the residual aldehyde groups were neutralized with 0.13 M glycine buffer and the grafts were freeze-dried. The grafts were inserted into individual envelopes and gas sterilized in ethylene oxide using a standard hospital procedure.

For CDI crosslinking, grafts were immersed in 1.1 ml of 20% BSA/125 I-BSA with 1 ml of 0.2 M CDI in phosphate buffer (0.5 M, pH 4.75). The coated prostheses were then processed as described above. The amount of chemically attached albumin on each segment was evaluated, either by gravimetry or by radioactivity counting, (LKB Rack Gamma II, LKB Bromma, Sweden) before washing.

Tests of degradation. The albuminated prostheses were inserted in sterile vials and then washed with constant stirring in a water bath shaker at 37°C. The incubating solutions were either phosphate buffered saline (PBS), bovine plasma, or pancreatin (0.1 g/l and 0.4 g/l), in solution with tris-(hydroxymethyl) aminomethane, HCl buffer at pH 7.6. Pancreatin (Sigma), was chosen because it contains a wide variety of hydrolytic enzymes including proteases and peptidases. Thus, the enzymes of pancreatin were capable of extensive hydrolytic action[14]. The experiments were done under sterile conditions to avoid any interference from microbial degradation.

Evaluation of the degradation rate. Individual coupons were withdrawn in triplicate from the solutions at 4, 24, 48, 72 and 144 h. After rinsing in distilled water, radioactivity counting was done for each segment in a LKB RACK Gamma II. Then the grafts were freeze-dried and weighed.

In vivo tests

Fabric selection. We selected a USCI Sauvage filamentous Dacron fabric (Bard Implants), which was cut into discs of 5 mm diam.

Albumin coating. We used the same procedure as for albuminating the prostheses except that the albumin was of rat origin (RSA: Sigma) containing 5 μCi of 125 I-RSA (NEN, Boston, Mass, USA).

Test of degradation. For the evaluation of the GA or CDI crosslinked albumin covered discs, 36 rats were selected and divided into 6 groups of 6 animals. The rats were anaesthetized with Halotane® (Hoechst Inc., Montreal, PQ, Canada) and prepared for surgery by shaving their abdominal regions and then preparing with betadine solution. Three discs were inserted into the peritoneal cavity with a 14 gauge needle and trocar.

Evaluation of the degradation. The discs were harvested at 1, 2, 7, 14, 21 and 28 d after implantation. The residual radioactivity was counted for each one.

Pathological analysis. The pathological state of the explanted albuminated grafts were studied by scanning electron microscopy according to the stablished protocol and by light microscopy[15]. Briefly, the explants were fixed in glutaraldehyde and dehydrated in a series of thanol dilutes (70, 90 and 100% ethanol). Then, the specimens were embedded in histor sin (LKB), cut into 3 μm sections with an ultracut (Reichert-Jung) and stained with methyl n blu .

RESULTS

In vitro degradation

The results f washing th coated grafts in PBS showed that after a v ry small removal f albumin, the level stabilized. After 144 h of shaking at 37°C, the loss of albumin in wov n or velour prostheses measured by weighing and radioactivity counting was less than 2%.

In plasma, the two methods of evaluation (gravimetry and radioactivity counting) gave contradictory results. Whereas we observed an increase in weight (5%) aft r plasma incubation, the radioactivity counting indicated a slight loss (2%). This might be due to the fact that plasma contains large amounts of proteins which can adsorb onto or diffuse into coated prostheses. In any case, there was no significant degradation of the albumin coating obtained by either GA or CDI crosslinking.

The removal of the chemically attached albumin in pancreatin solution (*Figure 1*) showed that the degradation rate of the coating depended upon the crosslinking ag nt. The GA crosslinked albumin degradation is linear with tim and depends upon pancreatin concentration. With the 0.1 g/l pancreatin solution, the loss of the coating was less than 30% at up to 144 h. This degradation was increased with higher pancreatin concentration. With 0.4 g/l, it was about 70% after 144 h of incubation. With CDI, the degradation was very rapid; at 24 h, the release of albumin coating was already 80%. It degraded totally at 144 h. W observed approximately the same rate of degradation either with 0.1 g/l or 0.4 g/l of pancreatin solution (*Figure 1a, b*).

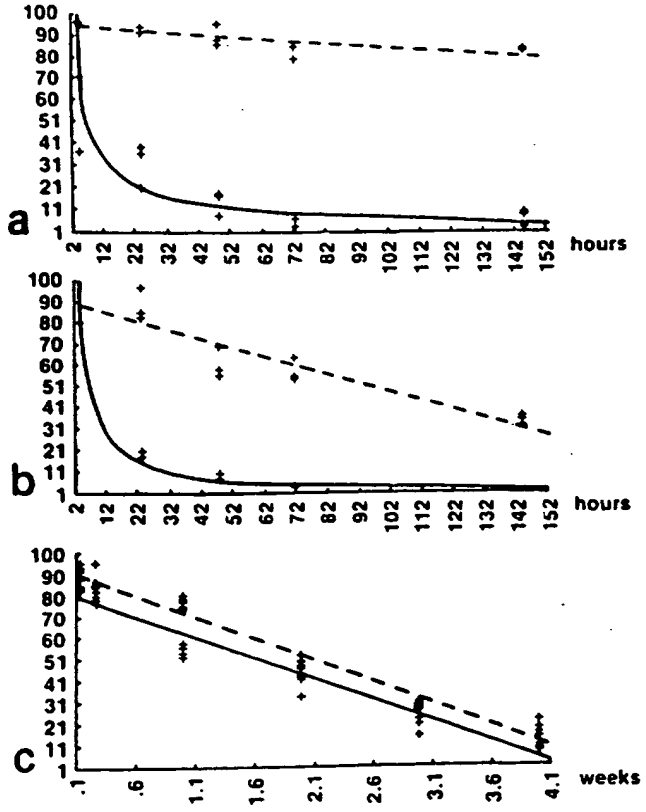

Figure 1 Percentage of crosslinked albumin still present in GA - - -, and CDI——, at various time intervals after in vitro exposure to pancreatin [(a), 0.1 g/l: (b), 0.4 g/l] or in vivo implantation in the peritoneal cavity of rats (c).

In vivo degradation

Degradation rate of albumin. Th results of th *in vivo* experim nts depicted in *Figure 1* show that:

(i) The two coatings are degradable *in vivo*;
(ii) Th removal of the albumin coating is rapid in the first 24 h, and then becom s lin ar with time;
(iii) All the attached albumin is r l ased at 5 wk;
(iv) The rate of degradation is approximately the same with either GA or CDI crosslinking.

Tissue reactions. The cellular reactions were rapid. At 24 h, the grafts were invaded by phagocytic cells. This cellular infiltration was seen even between the polyester yarns (*Figure 2 a, b*). Nevertheless, the CDI crosslinked grafts seemed more encapsulated by a dense, regular network of fibrillar material (*Figure 4 a, b*). After 1 wk, the fibrillar matrix density decreased and the granulocytic cells were found to be in contact with the albuminated polyester. At 4 wk, the GA crosslinked grafts were still infiltrated by a dense granulocytic tissue, with many giant cells (*Figures 3 a* and *5 a*). The tissue reactions with CDI crosslinked grafts were mild at that time and only rarely were granulocytes observed (*Figures 3 b* and *5 b*).

DISCUSSION

Radioactivity counting is more sensitive and reproducible for albumin assay than the gravimetric method, because it is specific for radi labelled albumin and does not include proteins adsorbed from the incubant in the assay.

The crosslinking mechanisms of glutaraldehyd and carbodiimid are different. Whereas GA crosslinks amino groups on th sam or different prot in molecul s by means of a chain mad of several of its own molecules[16], CDI activat s a carboxyl group to ultimat ly form a p ptidic bond with an amino group of the same protein (if st ric hindrance permits) or of another protein[17].

Since only peptidic bonds are present in coatings made by use of CDI, it is understandable that a protease mixture such as pancreatin is efficient in solubilizing it. In the case of GA, it is not known whether enzymes ar able to break the bonds formed, or if it is necessary to hydrolyse the proteins to release the untransformed chemical crosslink.

Since GA uses a chain of variable length to crosslink amino groups, it can more easily form intra- and intermolecular bonds. If the crosslink density is higher with GA than with CDI the proteases have more difficulty diffusing into the albumin mesh and the hydrolysis takes a long r time.

In vivo, the kinetics of the degradation of CDI crosslinked albumin is completely different to its *in vitro* counterpart and is similar to the degradation of th GA coating. On the one hand, *in vivo* proteolysis is effective, since GA albumin disappears at a rate approximately equivalent to the rate obtained *in vitro* with 0.2 g/l of pancreatin, yet on the other hand it is limited, as the degradation rate of CDI albumin is considerably reduced compared to that *in vitro*. This phenomenon is likely to be due

Figure 2 Histologic cross-section of the tissue response of the samples implanted for 24 h in the peritoneal cavity of rats. The methylene blue stainings illustrate the market nuclear cell reaction either with GA (a) or CDI (b) crosslinked albumin. (× 448).

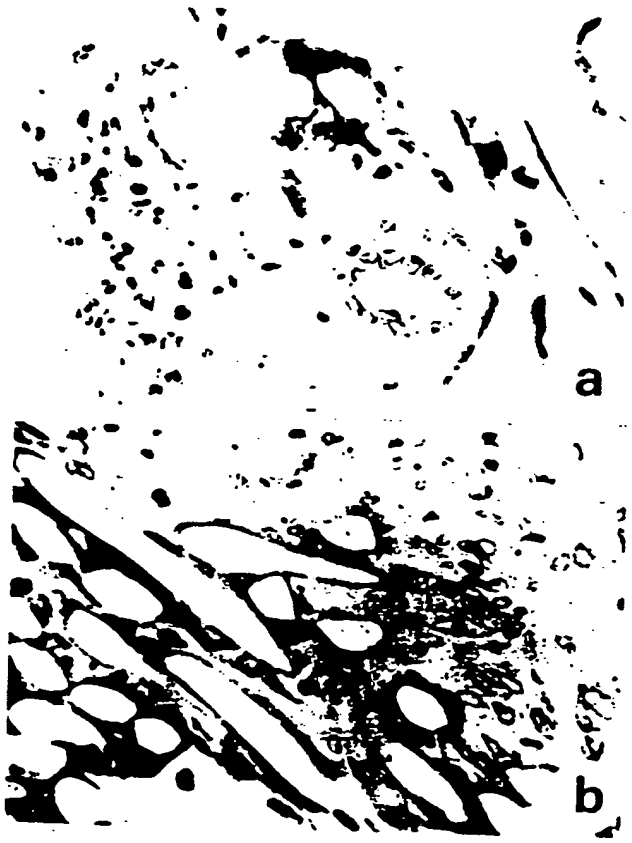

Figure 3 Histologic cross-section of the tissue response of the sample implanted for 28 d in the peritoneal cavity of rats. The methylene blue stainings illustrate a marked nuclear and giant cell reaction associated with the GA crosslinked albumin (a) compared to a mild cellular reaction with the CDI crosslinked albumin (b). (× 448).

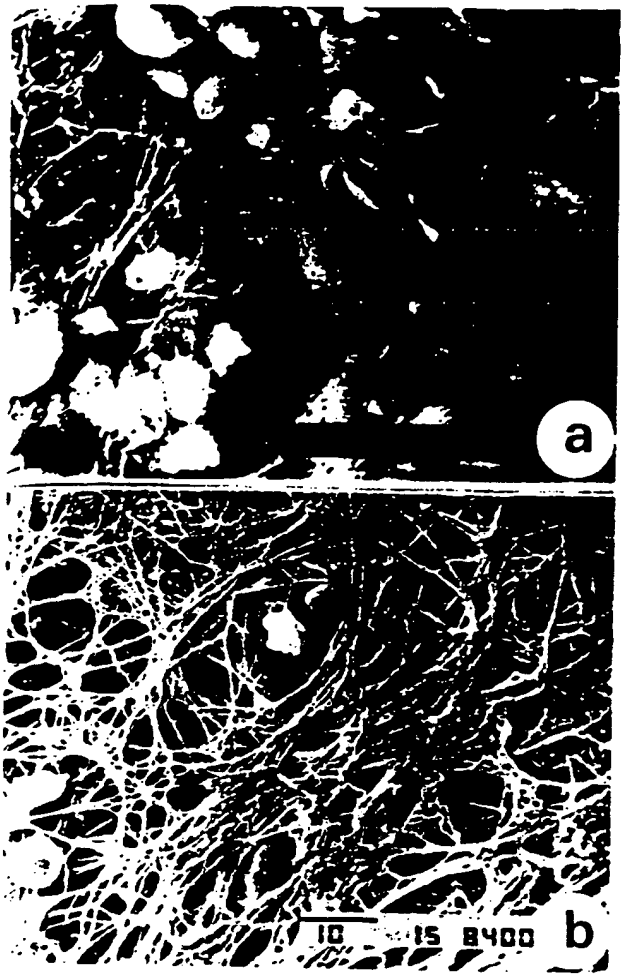

Figure 4 SEMs of the surface of the specimens 24 h after implantation showing a marked difference in the tissue response: the polyester coated with GA crosslinked albumin has a very small fibrillar network (a) compared to that of CDI crosslinked albumin (b).

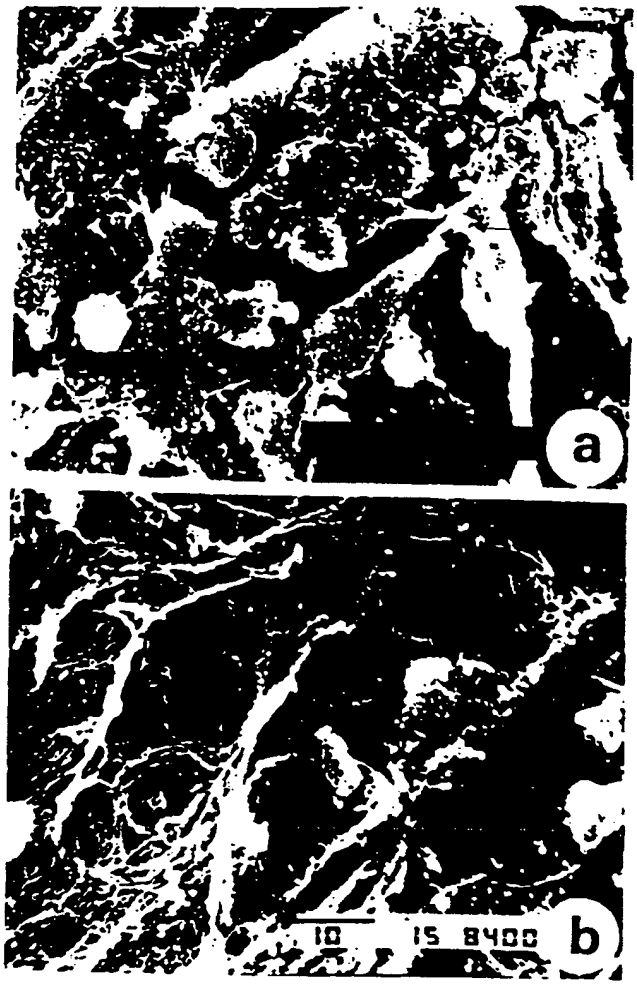

Figure 5 SEMs of the surface of the specimens 28 d after implantation. The tissue reaction caused by the GA crosslinked albumin is still very strong (a) whereas inflammatory cells are no longer visible on the CDI crosslinked albumin (b).

to the differences between degradation mechanisms, namely, pancreatin *in vitro* and phagocytic cells *in vivo*. The pathological analysis showed two different patterns of cellular reaction depending upon the crosslinking agent used. Grafts coated with CDI albumin contained mainly neutrophilic cells (acute inflammation type) and the tissue response was very mild 4 weeks after implantation. The ease of proteolysis or the presence of only amino acid after degradation might be responsible for this. In contrast, the tissue response observed in GA albumin grafts involved macrophages and foreign body giant cells (chronic inflammation type) and was still very active 4 weeks after implantation. The difficulty of proteolysis and the specific action of GA-derived crosslinks are the two factors suspected to account for this difference.

CONCLUSION

The present work demonstrates the stability of the albumin crosslinking ither by GA or CDI in PBS and plasma, and its biodegradability by enzymatic hydrolysis. Th *in vivo* behaviour of CDI crosslinking c mpared to that of GA crosslinking combined with the absenc of residual ch micals aft r hydrolysis and of a chr nic inflammatory reaction speak for the use of th former for therapeutical applications.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada (grant MA 6764). The authors are indebted to J. Bastien, S. Bourassa, H. Desbiens, D. Gagnon, D. Mart l, L. Martin, R. Paynter and C. Mongrain for valuabl discussions and technical assistance. The gift of prostheses and fabrics by Bard Implants Division is gratefully acknowledged.

REFERENCES

1 Yates, S.G., Barros d'Sa, A.A.B., Berger, K., Fernandez, L.G., Wood, S.J., Rittenhouse, E.A., Davis, C.C., Mansfield, P.B. and Sauvage, S.L., The preclotting of porous arterial prostheses, *Ann. Surg.* 1978, **188**, 611-622

2 Topping, C., Guidoin, R., Roy, J., Picard, R., Bernier, J., Gosselin, C., Blais, P., Martin, L., Marois, M. and Noël, H.P., La précoagulation des prothèses vasculaires en Dacron tissé, *J. Chir.* 1980, **117**, 403-409

3 Wooster, D.L., Krejden, S. and Louch, R.E., Intraoperative bacterial contamination of vascular grafts, *Roy. Coll. Phys. Surg. Can. 53rd Annual meeting*, Montreal, Sept., 1984

4 Gloviczki, P., Hollier, L.H., Hoffman, E.A., Vlabe, G., Trasbek, V.F. and Kaye, M.P., The effect of preclotting on surface thrombogenicity and thromboembolic complications of Dacron grafts in the canine thoracic aorta, *J. Thorac. Cardiovasc. Surg.* 1984, **88**, 253-258

5 Bascom, J.V., Gelatin sealing to prevent blood loss from knitted arterial grafts, *Surgery* 1961, **50**, 504-512

6 Humphries, A.W., Hawk, W.A. and Cuthbertson, A.M., Arterial prosthesis of collagen impregnated Dacron tulle, *Surgery* 1961, **50**, 947-954

7 Jordan, G.L., Stump, M.M., Allen, J., DeBakey, M.E. and Halpert, B., Gelatin impregnated Dacron prosthesis implanted in porcine thoracic aorta, *Surgery* 1963, **53**, 45–49

8 Chvapil, M. and Krajicek, M., Use of collagen in the construction of an arterial prosthesis, *Med. Trends Vasc. Surg.* 1970, **1**, 120–140

9 Packman, M.A., Evans, G., Glynn, M.F. and Mustard, J.F., The effect of plasma proteins on the interaction of platelets with glass surface, *J. Lab. Clin. Med.* 1969, **73**, 686–697

10 Gyurko, G.Y., Szücs, J., Banhegyi, J., Kenyeres, I., Szikszai, P., Noviczki, M. and Posze, G., Experimental experience with polyester velour in vascular plastic surgery, *Acta Chir. Acad. Sc. Hung.* 1974, **15**, 35–43

11 Domurado, D., Thomas, D. and Broun, G., A new method for producing proteic coatings, *J. Biomed. Mater. Res.* 1975, **9**, 109–110

12 Domurado, D., Guidoin, R., Marois, M., Martin, L., Gosselin, C. and Awad, J., Albuminated Dacron prostheses as improved blood vessel substitutes, *J. Bioeng.* 1978, **2**, 79–91

13 Guidoin, R., Snyder, R., Martin, L., Botzko, K., Marois, M., Awad, J., King, M., Domurado, D., Bedros, M. and Gosselin, C., Albumin coating of a knitted polyester arterial prosthesis: an alternative to preclotting, *Ann. Thorac. Surg.* 1984, **37**, 457–465

14 Needham, D.K., Buchanan, D.R., Gupta, B.S. and Horton, H.R., Enzymatic removal of formalin-fixed tissues from polyester arteries and its effect on physical properties of fibers, *J. Biomed. Mater. Res.* 1982, **16**, 659–668

15 Guidoin, R., Gosselin, C., Martin, L., Marois, M., Laroche, F., King, M., Gunasekera, K., Domurado, D., Sigot-Luizard, M. -F. and Blais, P., Polyester prostheses as substitutes in the thoracic aorta of dogs. I. Evaluation of commercial prostheses, *J. Biomed. Mater. Res.* 1983, **17**, 1049–1077

16 Monsan, P., Puzo, G. and Mazarguil, H., Etude du mécanisme d'établissement des liaisons glutaraldéhyde-protéines, *Biochimie* 1975, **57**, 1281–1292

17 Sheehan, J.C. and Hlavka, J.J., The cross-linking of gelatin using a water soluble carbodiimide, *J. Am. Chem. Soc.* 1957, **79**, 4528–4529